AD-A069 512 MARYLAND UNIV COLLEGE PARK DEPT OF MICROBIOLOGY F/G 6/13
MICROBIAL ECOLOGY STUDIES OF BIOFOULING OF TREATED AND UNTREATE--ETC(U)
DEC 78 R R COLWELL, A ZACHARY, D ALLEN N00014-75-C-0340
UNCLASSIFIED UM/ONR-5 NL

1 OF 1
AD
A069512

END
DATE
FILMED
7-79
DDC

LEVEL II

(12) (B S)

Annual Report

submitted to

OFFICE OF NAVAL RESEARCH

(6) Microbial Ecology Studies of Biofouling of Treated and Untreated Wood Pilings in the Marine Environment.

(15) Contract No. N00014-75-C-0340 P00004

Task No. NR 133-081

(9) Annual Report, (14) No. UM/ONR-5

1 Jan - 31 Dec 78,

by

(10) R. R. Colwell,
Department of Microbiology
University of Maryland
College Park, Maryland 20742

Assisted by

A. Zachary,
D. Allen,
M. R. Belas
B. Austin
K. Smith



Period covered: January 1, 1978 - December 31, 1978

(11) 31 Dec 78 (12) 26p.



| Accession For | |
|---|---|
| NTIS GRA&I | ☑ |
| DDC TAB | ☐ |
| Unannounced | ☐ |
| Justification | |
| By | |
| Distribution/ | |
| Availability Codes | |
| Dist | Avail and/or special |
| A | 23 |



407 304

LB

## INTRODUCTION

Aquatic bacteria are a significant component of the ecosystem in the natural environment. Bacteria of ecological significance in the marine environment include those that are primary colonizers of natural substrates such as sand grains, (Meadows & Anderson, 1968), degraders of complex organic compounds, e.g., petroleum (Mulkins-Phillips & Stewart, 1974), naphthalene (Raymond, 1974), and pesticides (Gibson & Brown, 1975), as well as transformers of heavy metals (Nelson & Colwell, 1975), which are often antibiotic resistant (Allen et al., 1977), and contributors to the food chain (Berk et al., 1976). The establishment of a primary microbial community on substrates, is a prerequisite for subsequent attachment of marine macroorganisms viz. barnacles, algae, etc. (Crisp, 1974). In brief, they are the primary agents of mineralization and biological colonization in the oceans, coastal waters and estuaries.

Study of biological colonization of pilings associated with biodegradation and biodeterioration of naphthalene and naphthalene creosote-treated wood has been a focus of research work on this project during the past year. The pilings at Pier 3 at the Roosevelt Roads Naval Station, Puerto Rico were replaced in late 1977 with high naphthalene creosote-treated wood. Field work at Roosevelt Roads, was carried out to determine the effect of creosote and creosote/naphthalene from the pilings on the microbial ecology of the water and sediment in the area of pier 3 at Roosevelt Roads. An objective of this research project was to determine the microbial species structure and diversity of the natural flora of water, sediment, and fauna, in particular, the gut flora of the wood borers, before and after the installation of the treated pilings. Environmental effects of naphthalene and naphthalene-treated wood [illegible] ecological succession leading to establishment of a fouling community on the pilings, the ability of the natural microbial communities

to biodegrade treated and untreated wood exposed to water and sediment, and the effect of the naphthalene and naphthalene/creosote on the commensal flora of wood-boring organisms were studied. The effects of petroleum, in enhancing or inhibiting the biodeterioration of treated and untreated pilings, was also considered.

Specific objectives of the research in the initial phase of the project have been achieved. Preliminary work on the biodegradation of naphthalene, creosote, naphthalene-enriched creosote, and petroleum has been done. The bacterial flora of beach sand, wood pilings, and Limnoria tripunctata samples collected at Roosevelt Roads was analyzed and changes in the bacterial community structure following implantation of new naphthalene/creosote treated wood pilings was evaluated. Bacteria associated with the pilings at the Navy Base were examined to determine the effect of naphthalene-enriched creosote on bacterial attachment and colonization. Selected bacterial species isolated from the pilings in Puerto Rico were used in laboratory experiments to elucidate mechanisms by which these bacteria attach to wood surfaces.

## MATERIALS AND METHODS

### Collection of samples

Water and sediment samples were collected aseptically from two locations within the U.S. Naval Base at Roosevelt Roads, Puerto Rico, situated on the eastern extreme of Puerto Rico in the Caribbean Sea (Fig. 1). One sampling site is located within the dockyard at the main pier (Pier 3) and is actively used by oceangoing vessels. Salinity of the water at this site is 35 $^{o}/_{oo}$. At the time of the initial sampling the temperature of the water was 29$^{o}$C. One side of the pier is constructed from creosote-impregnated

wooden pilings. About one-third of the pilings had been installed within three weeks of the sampling, starting in late summer and continuing through the fall of 1977. At the time of the October sampling, construction was in progress. On the other side of the pier, the pilings are in an advanced state of deterioration, resulting from attack by the wood-boring isopod, Limnoria tripunctata. The pilings have been destroyed principally at the air-water interface, with dramatic destruction of the wood. A control site was selected for the study, located 5 m from the shore line on a deserted beach, approximately 5 km from Pier 3, on the Atlantic side of the island. Water salinity and temperature at the time of the sampling in October 1977, were 35 °/oo and 30°C, respectively. Water samples were collected 1 m below the surface using sterile polypropylene bottles. Additional samples for chemical analyses were collected in 500 ml aliquots in sterile bottles and filtered through sterile cellulose filters to remove particulate matter. The filtrate was mixed with 100 ml of benzene, shaken for one minute, allowed to settle, and the benzene fraction separated and stored at 4°C. Sediment samples from the control site were collected in polypropylene bottles and at the base of the old and new pilings and 3 m from the base of the pilings at Pier 3. Wood samples for microbiological analyses were also collected. These were cut from the pilings at the air-water interface and 1 m above and below the air-water interface. Both old and new pilings were sampled. Assistance of Navy SCUBA divers stationed at Roosevelt Roads permitted manual collection of samples at the pier, minimizing disturbance of the water, sediment and wood surfaces for subsequent microbiological and chemical analyses.

Samples of wood, 40% naphthalene-enriched creosote-treated wood (supplied by Dr. David Webb, Koppers Co.) glass and oyster shell, after exposure for 5 days at either the Pier 3 or control site were subjected to microbiological analyses.

Bacteriological Analyses

Samples for bacteriological analyses were examined within 15 min of retrieval. Other samples were stored at 4°C. Serial dilutions of sediment, water, and wood were prepared to $10^{-5}$, $10^{-3}$, and $10^{-3}$, respectively, using 9 ml aliquots of marine salts solution (2.4% (w/v) NaCl, 0.7% (w/v) $MgSO_4$ $7H_2O$, 0.075% (w/v) KCl). Aliquots (0.1 ml) of the diluted samples were pipetted onto plates of 2216 agar (Difco). Three replicates of each dilution were plated and the inoculated plates were incubated for ca. 27°C for 5 days. In addition, 2216 agar for isolation of bacteria was prepared and directly streaked with test surfaces (wood, 40% naphthalene/creosoted treated wood, glass and oyster shell) exposed at Pier 3 or the control site. Plates containing between 30 and 300 colonies were used for random selection of colonies for subsequent streaking onto plates of 2216 agar, with three subsequent serial streakings of each culture being done to ensure purity of the strains.

Heat fixed smears of the pure cultures were stained using Hucker's modification of the Gram stain (Hucker & Conn, 1923). After purification, the cultures were inoculated onto 2216 agar slopes. Any culture suspected to be mixed was successively re-streaked until pure cultures were recovered, as determined by examination of colonial morphology and phase contrast microscopy. Original isolates were maintained in stock culture on 2216 agar slopes at 4°C. Subcultures of the working bench cultures were prepared every 12 weeks. The 272 isolates carried through the full testing series are listed in Table 1.

Each strain was examined for 19 biochemical, cultural, morphological, nutritional and physiological characters. (Table 2) Media were inoculated from 48 hr cultures grown on 2216 agar and were incubated at 25°C

for 7 days. Whenever possible, 2216 agar was used as the basal medium for the test media. Otherwise, media were prepared in marine salts solution. To assess possible test error, 10 strains were examined in duplicate, otherwise tests were carried out once and were repeated only in the event of inconclusive results. The tests used in the analysis have been described previously (Colwell & Weibe, 1970; Austin et al., 1977).

Coding of Data

The characters were coded '1' for positive or present, '0' for negative or absent, and '9' for non-compatible or missing.

Identification of the Phenetic Groups

Phena were identified using a diagnostic dey developed during previous studies at Roosevelt Roads under this contract (Austin et al, 1979) along with Bergey's Manual of Determinative Bacteriology (Buchanan & Gibbons, 1974) and the tables of Cowan (1974).

Electron Microscopy

1) Discs of untreated wood, and wood impretnated with 10% and 40% naphthalene-containing creosote cover slip glass, and oyster shell were mounted on aluminum scanning electron microscope (SEM) stubs and these were suspended on a frame 1m below the water's surface at the Pier 3 sampling site (Zachary, Taylor, Scott & Colwell, 1978). Samples of each wood type were removed from the water after exposure to the water for 2 and 4 days. The samples were immediately fixed in a 2% (w/v) buffered gluteraldehyde solution (pH 7.4), and subsequently prepared for SEM observation, including ethanol dehydration, critical point drying under carbon dioxide, and sputter coating with gold-palladium alloy.

2) Electron microscopy of microoganisms associated with Limnoria tripunctata.

Limnoria tripunctata specimens were collected from Limnoria-infected wood at Pier 2 and placed in 2% buffered gluteraldehyde containing ruthenium red prepared with artificial seawater.

Whole specimens of L. tripunctata and dissected digestive tracts, after fixation for 18-24 hours (4°C), were prepared for microscopic examination, following the procedures described above. After fixation in gluteraldehyde, the digestive tracts were fixed for an additional 18-24 hours at 4°C, in 0.1 M cacodylate buffered (pH 7.4) 1% (w/v) osmium tetroxide.

Fixed isopods and gut samples were dehydrated, using a graded acetone series and embedded, in known orientation, in Epon 812 epoxy plastic. Thin sections cut through the plastic-embedded digestive tracts were stained with uranyl acetate and lead citrate and examined using a Hitachi HU 11-A electron microscope. Thick sections cut from the same embeddings were stained with Safranin and examined using phase contrast light microscopy.

Laboratory studies of attachment

A pure culture of the bacterial strain OWD-1 was previously isolated from the surface of the old wooden pilings. This strain was used in a series of laboratory experiments in which the bacteria were allowed to attach to glass surfaces. Attachment was monitored using thin glass discs mounted on scanning electron microscope (SEM) stubs with epoxy adhesive. The stubs were sterilized with ethanol, flamed, and placed in flasks containing a sterile, dilute nutrient medium (0.01% w/v peptone in marine salts solution). Bacterial strain OWD-1 was inoculated into flasks and incubated at 25°C up to 48 hr. At the end of each incubation period, stubs were removed from the flasks and fixed in a 2.5% gluteraldehyde-

marine salts solution buffered to pH 7.4 with 0.2 M sodium cacodylate. The stubs were allowed to remain in the fixative held at 4°C for 24 hr. Stubs were subsequently prepared for SEM visualization using dehydration with a graded ethanol series, critical point drying under $CO_2$, and sputter coating with gold-palladium alloy. Either a Cambridge Mark III or ISI-60 scanning electron microscope was employed to examine the specimens.

Transmission electron microscopy

Bacterial cultures were grown at 25°C on Z broth (1% w/v Bacto-tryptone; 0.1% w/v Glucose; 0.1% w/v Yeast Extract in a marine salts base) to a concentration of approximately $10^8$ cells/ml. A drop of the bacterial culture was placed on carbon-reinforced, formvar-coated copper transmission electron microscope (TEM) grids. These were fixed with gluteraldehyde fixative and air-dried. Samples were negatively stained, using 2% uranyl acetate, dried, and examined using an RCA EMU-3 transmission electron microscope.

## RESULTS AND DISCUSSION

Taxonomy and ecology of the marine bacterial flora of the water, sediment and pilings.

In previous studies carried out in October 1977 under this contract, the bacterial microflora of samples collected at Roosevelt Roads and vicinity, were subjected to taxonomic analyses using 107 biochemical, physiological, morphological and nutritional tests (Austin et al., 1979 ). Based on results of the studies of the first year of this project 31 taxonomic clusters, or phena, were established, and 5 major phena were identified (comprising 58% of the isolates). A diagnostic key was developed for the microflora at Roosevelt Roads, by selecting 19 characters of the 107 examined, which were found to be essential for distinguishing the 31 phena originally identified (Table 2 ). Use of such a diagnostic key allowed more rapid identification of the marine bacterial isolates obtained in this research project.

In the work done in June 1978, the same sampling sites in Puerto Rico were examined to determine if changes in the microbial community structure had occurred, due to seasonal conditions and/or the presence of the new naphthalene-enriched creosote pilings implanted at Pier 3. Results show that the basic bacterial community structure has not changed significantly, with only one exception. The same four major bacterial phena were observed (comprising 55% of the isolates) (Table 3 ). This observation also indicated the validity of the diagnostic key for identification of the bacteria at Roosevelt Roads.

In our sampling we discovered 10 strains of bacteria which could not be classified into existing phena. We have listed these in five new phena, 32-36 in Table 1, and have listed the characteristics of these new phena in Table 4.

The significance of the differences observed in the number of bacterial isolates in the four major phena between the October and June samplings most likely arises from sampling variation, or may represent shifts in the bacterial populations due to changes in seasonal conditions (e.g. water temperature, rainfall, etc.) or changes occurring in response to the presence of new pilings and continuing development of a biological fouling community on the pilings. Continued study of the microflora at Roosevelt Roads will aid in identifying which of these factors is the predominant influence on the microbial populations.

A significant finding is the observation that three of the major phena viz. 4, 22 and 17, are clearly characteristic of the Pier 3 micro-habitat and are only rarely found at the Control Site, indicating that a distinct microbial population is present at Pier 3. Furthermore, the Pier 3 microbial community structure has not been totally altered by implacement of naphthalene-enriched wood pilings.

In the October sampling, the predominance of Bacillus sp. group 13, as well as groups 14, 15, 27, 28 and 29, was considered to be unusual, as these bacteria are not generally a major component of marine bacterial communities, but more often are terrestrial, i.e. soil, microorganisms. Bacillus spp. produce spores which are resistant to environmental extremes and can be transported into and remain viable outside of the soil habitat.

It was hypothesized that the large number of Bacilli found at the Control Site in the October, 1977 sampling represented spores, not metabolically active cells, which were washed into the marine waters surrounding Roosevelt Roads by the heavy rains which occurred at the base for two weeks prior to our sampling trip.

The complete absence of groups 13, 14, 15 and the isolation of only 1 Bacillus species at Pier 3, and only 4 strains of Bacillus at the Control Site in June, 1978, supports this hypothesis. If Bacillus spp. were a component of the microflora, then they would be found at all times of the year.

Results of taxonomic analyses of bacteria isolated from surfaces of 40% naphthalene/creosote-treated wood, glass, and oyster shell exposed to microbial colonization for 5 days at Roosevelt Roads, revealed that most of the bacteria attaching to the surfaces were not identical to those in the surrounding water, sediment or pilings. These organisms represent the pioneer species, which first attach to surfaces, and are later replaced by other types of bacteria, as the colonization succession proceeds. It is intended to continue study of the primary colonizers to determine their specialized characteristics.

In October 1977, it was observed that a very low diversity of bacterial community structure occurred on the newly emplaced pilings. Such low diversity is characteristic of newly forming biological communities (e.g. forming on new surfaces of the pilings) or stressed communities (e.g. addition of allocthonous material, such as naphthalene). In June 1978, the low diversity pattern had changed and, instead, an increased diversity was noted for the bacterial community at Pier 3. Thus, the formation of a mature biofouling community appears to be occurring on the pilings. The data suggest that the effects of the placement of new, naphthalene-enriched creosote pilings at Pier 3 has only a short-term impact on the microbial ecology, which is localized in the vicinity of the Pier. The pilings installed at Roosevelt Roads do not, therefore, appear to pose a long-term threat to the ecological balance of marine microbial communities in the vicinity of the Roosevelt Roads harbor area.

Electron microscopy

The short exposure periods, i.e., 2 to 5 days, were sufficient to allow microbial colonization of all the test surfaces, indicating that microfouling is an extremely rapid process in the tropical marine environment at Puerto Rico. Although all surfaces were colonized, the rapidity and extent of microfouling was greater on the untreated wood than on the naphthalene/creosote-treated wood, but no differences in microfouling were evident between 10 and 40% naphthalene/creosote-treated wood. After 2 days in the water, the untreated wood surface was covered by extensive colonies of floc-forming bacteria, which appeared as mat-like coverings at low magnification, but were clearly visible as bacteria enmeshed in an extensive fibrillar network, when viewed at higher magnification. In contrast, on the naphthalene/creosote-treated wood, at 2 days, bacteria demonstrating only a few fibrils were observed and extensive mat-like colonies were not yet visible. After 4 days in the water, however, large mat-like colonies of floc-forming bacteria were evident on the naphthalene/creosote-treated wood. These observations confirmed our early findings that microbial succession is similar on treated and untreated wood, but colonization occurs more slowly on naphthalene/creosote-treated wood surfaces.

The more rapid microfouling of untreated wood was clearly evident from heavy bacterial growth and presence of large numbers of diatoms observed after exposure of the wood for 5 days in the water. As in previous studies, diatoms were not observed to occur within this time period on the naphthalene/cresote-treated wood. Naphthalene/creosote treatment appears to have a specific inhibitory or toxic effect on eucaryotic diatom cells, but not on procaryotic bacterial cells.

The floc-forming bacteria observed on the wood surfaces were rarely seen on glass or oyster shell surfaces. The predominant type of microorganism on the latter surfaces was diatoms. Thus, a specific bacterial flora appears to be involved in colonization of wood surfaces. The predominance of diatoms on glass and oyster shell is in sharp contrast to the absence of these microorganisms on naphthalene/creosote-treated wood, providing further support of a specific inhibitory effect of naphthalene-enriched creosote on the attachment of diatoms to the pilings.

Scanning electron microscopy

Within 3 hr after inoculation of dilute nutrient medium with bacterial strain OWD-1, numerous aggregates of bacterial cells were observed to adhere to the surface of the glass disc epoxied to the SEM stub. Most of the aggregates showed, concommitant with attachment, thin extracellular fibrils, which extended from the cell surface outwards towards the glass surface. These fibrils extended outward to ca. 2.5 μm in a peritrichous arrangement, with an average of ten fibrils/cell.

At 24 hr, the fibrils were abundant, extending from the individual cells to the glass surface, as well as from one cell to another. By 48 hrs, the fibrillar network had increased to an extensive mat composed of many single fibrils. The mat of fibrils covered areas of the glass surface, with bacteria adhering to, as well as being embedded within, the network. These bacterial colonies, enmeshed in a fibrillar mat, were similar to those observed in the field studies.

Transmission electron microscopy

Negatively stained cells of strain OWD-1 exhibited several extracellular structures when visualized by transmission electron microscopy. The cells possessed a single polar, sheathed flagellum and, in addition, many thinner fibrillar structures which were less than half the diameter of the flagellum and much more numerous (~50-100 fibrils/cell). The fibrils appeared to be

flexible, displaying various forms on the dried specimen, unlike the flagellum which possessed the characteristic wave pattern associated with that structure. The lengths of the fibrils could not be accurately measured, due to aggregation, clumping and possible breakage of the structures.

The fibrillar strands observed on OWD-1 may represent one form of attachment structure which aids the bacterium in its attachment to submerged aquatic surfaces. That these structures appear to proliferate as the organism settles on, and attaches to the glass surface, indicates a correlation between the events although the actual mechanism of attachment of strain OWD-1 is not yet fully characterized. Chemical analyses of the nonflagellar, extracellular appendages is underway. Preliminary evidence indicates a proteinaceous nature of these fibrils.

Previously, our studies, using electron microscopy, showed that microorganisms were not found in the digestive system of L. tripunctata reared on untreated pine wood blocks in a laboratory aquarium. The surfaces of the exoskeleton of the isopod were, however, subject to bacterial colonization.

Extensive microscopic examination of L. tripunctata collected from the pilings at Roosevelt Roads, was carried out, showing that contrary to isopods reared on untreated wood, the L. tripunctata from creosote-treated wood pilings in Puerto Rico did have microorganisms present in their digestive systems. Bacteria, which colonize surfaces and tunnels produced by the Limnoria, are ingested, along with wood fragments, as the isopod bores into the pilings. The gut contents, including the ingested bacteria, are encased in a peritrophic membrane which, in effect, isolates the ingested material from the intestinal lining. Although bacterial cell debris is occasionally observed, most of the ingested bacteria appear to be intact.

A distinct group of bacteria were also found outside the gut contents in the peritrophic space, i.e., the space between the peritrophic membrane and the intestinal lining. The bacteria were observed to be in close association with the intestinal epithelium, and were often attached by capsular fibrils to the gut wall, in invaginations of the intestinal lining. These gut-associated bacteria were also present in isopods which contained no ingested food material. The gut-associated bacteria were found throughout the intestinal tract, generally in low numbers (1-5 cell profiles/thin section), with one exception noted in a specimen in which the peritrophic membrane was not completely intact. In this case, gut-associated bacteria were present in much greater abundance, with most cells coating the intestinal lining and some cells attached to the periphery of the gut contents.

There were no discernable signs of damage to the host tissues associated with the presence of the gut-associated bacteria, suggesting that these bacteria may not be pathogenic for the isopod.

The presence of gut-associated bacteria in all the L. tripunctata specimens examined from the creosote treated pilings at Roosevelt Roads, the close association of the bacteria to the gut lining (i.e., in gut invaginations), and the lack of discernable pathogenic effects on the isopod, raises the possibility of a commensal relationship between the gut bacteria and the isopod. Many of the gut-associated bacteria contained electron-transparent cytoplasmic inclusions. This type of inclusion is characteristic of a wide variety of bacteria grown on hydrocarbons (Scott and Finnerty, 1976). The inclusions, in the latter case, contain hydrocarbons ingested by the bacterium from the growth medium (Kennedy et al., 1975). It is possible, but yet to be proven, that uptake and/or utilization of hydrocarbons by the gut-associated bacteria in L. tripunctata from creosote-treated wood can produce a detoxification of the creosote for the isopod. This type of commensal relationship,

whereby acquired gut microorganisms detoxify ingested creosote, may, in part, explain the resistance to creosote attributed to this tropical isopod species which causes severe damage to creosote-preserved wood structures in Puerto Rican and other tropical waters.

Examination of the exoskeleton of L. tripunctata specimens from the creosote-treated pilings at Roosevelt Roads, revealed the presence of large numbers of bacteria adhering to its external surfaces. Some cells were attached singly by capsular fibrils, but, quite often, several bacterial cells were observed in a common capsular mass, extending several micrometers from the exoskeletal surface. Many of the bacteria attached to the exoskeleton possessed electron-transparent cytoplasmic inclusions, similar to those observed in bacteria grown on hydrocarbons, suggesting that hydrocarbon uptake and/or utilization leading to creosote detoxification for Limnoria may be facilitated by exoskeleton bacteria, as well as gut-associated bacteria of the isopod. Future studies will be aimed at isolating and characterizing the gut-associated, and exoskeleton-associated bacteria of L. tripunctata to determine whether a commensal relationship, resulting in creosote-resistance for Limnoria, exists between these microorganisms and the isopod.

This research was supported in part by the Office of Naval Research, Microbiology Program, Naval Biology Project, under Contract No. N00014-75-C-0340, NR133-081. We are grateful for the excellent assistance provided during this study by the Resident Officer in Charge of Construction, and the U.S. Navy divers at Roosevelt Roads, P.R.

# LITERATURE CITED

Allen, D.A., B. Austin, and R.R. Colwell. 1977. Antibiotic Resistance Patterns of Metal-Tolerant Bacteria Isolated from An Estuary. Antimicrobial Agents and Chemotherapy 12:545-547.

Austin, B., D.A. Allen, A.L. Mills, and R.R. Colwell. 1977. Numerical taxonomy of heavy metal-tolerant bacteria isolated from an estuary. Can. J. Microbiol. 23: 1433-1447.

Austin, B., D.A. Allen, A. Zachary, M.R. Belas, and R. R. Colwell. 1979. Ecology and taxonomy of bacteria attaching to wood surfaces in a tropical harbor. Can. J. Microbiol. (in press).

Berk, S., R.R. Colwell, and E. Small. 1976. A study of feeding responses to bacterial prey by estuarine ciliates. Trans. Am. Microscop. Soc. 95:514-520.

Buchanan, R.E. and N.E. Gibbons (eds.). 1974. Bergey's Manual of Determinative Bacteriology, 8th ed. The Williams and Wilkins Co., Baltimore.

Colwell, R.R. and W.J. Weibe. 1970. "Core" characteristics for use in classifying aerobic, heterotrophic bacteria by numerical taxonomy. Bull. Georgia Acad. Sci. 28:165-185.

Cowan, S.T. 1974. Cowan and Steel's Manual for the Identification of Medical Bacteria. Cambridge University Press.

Crisp, D.J. 1974. Factors influencing the settlement of marine invertebrate larvae. p. 177-265 in P.T. Grant and A.M. Macke (ed.), Chemoreception in Marine Organisms. Academic Press Inc., New York.

Gibson, W.L. and L.R. Brown. 1975. The metabolism of parathion by Pseudomonas aeruginosa. Dev. Indust. Microbiol. 16:77-87.

Hucker, G.J. and H.J. Conn. 1923. Methods of Gram Staining. Technical Bulletin of the New York State Agricultural Experimental Station, No. 93.

Kennedy, R.S., W.R. Finnerty. K. Sundarsanan and R.A. Young. 1975. Microbial assimilation of hydrocarbons. I. The fine structure of a hydrocarbon oxidizing Acinetobacter sp. Arch. Microbiol. 102:75-83.

Meadows, P.S. and J.G. Anderson. 1968. Micro-organisms attached to marine sand grains. J. Mar. Biol. Assoc. U.K. 48:161-175.

Mulkins-Phillips, G.J. and J.E. Stewart. 1974. Distribution of hydrocarbon utilizing bacteria in north-western Atlantic waters and coastal sediment. Can. J. Microbiol. 20:955-962.

Nelson, J.D. and R.R. Colwell. 1975. The ecology of mercury-resistant bacteria in Chesapeake Bay. Microbial Ecol. 1:191-218.

Raymond, D. 1974. Metabolism of methylnaphthalenes and other related aromatic hydrocarbons by marine bacteria. Ph.D. Thesis, Rutgers University

Scott, C.C.L. and W.R. Finnerty. 1976. A comparative analysis of the ultra-structure of hydrocarbon-oxidizing micro-organisms. J. Gen. Microbiol. 94:342-350.

Zachary, A., M.E. Taylor, F.E. Scott, and R.R. Colwell. 1978. A method for rapid evaluation of materials for susceptibility to marine biofouling. Int. Biodeterior. Bull. 14:111-118.

Table 1. Bacteria isolated from Roosevelt Roads during sampling trip in June 1978. Numbers in parentheses represent strains isolated at source indicated.[1]

| Phenon | Total Number of Strains | Source |
|---|---|---|
| 1 | 2 | CS(1), Psh(1) |
| 2 | 30 | SCR(2), OWD(2), NWD(1), PW(2), CBS(1), CS(4), P40(3), Psh(2), Csh(2), $CS_{50}$(1), $CS_{100}$(7), $CW_{100}$(1) |
| 3 | 2 | CW(1), $CW_{50}$(1) |
| 4 | 48 | 1↑(16), SCR(12), 40%(9), 10%(1), PS(1), CBS(5), $CS_{100}$(3), CW(1) |
| 5 | 3 | PS(1), $CW_{100}$(2) |
| 6 | 2 | 40%(1), PW(1) |
| 7 | 2 | CBS(2) |
| 8 | 7 | 10%(1), 40%N(1), OWD(2), $CW_{100}$(3) |
| 9 | 0 | |
| 10 | 4 | NWD(1), PW(2), Cg(1) |
| 11 | 0 | |
| 12 | 1 | CW(1) |
| 13 | 0 | |
| 14 | 0 | |
| 15 | 0 | |
| 16A | 7 | 10%(3), NWD(1), PW(1), PS(2) |
| 16B | 0 | |
| 17 | 40 | 1↓(14), 40%(5), 10%(1), 40%N(4), OWD(3), NWD(9), PW(2), PS(2) |
| 18 | 0 | |
| 19 | 4 | 10%(2), PW(1), $CW_{100}$(1) |
| 20A | 7 | NWD(3), PW(2), PS(2) |
| 20B | 4 | SCR(2), 10%(1), CBS(1), Pg(1) |
| 21 | 1 | CBS(1) |
| 22 | 35 | 1↑(2), 10%(6), 40%N(12), OWD(5), NWD(2), CS(1), CW(4), $CW_{50}$(3) |

Table 1. (continued)

| Phenon | Total Number of Strains | Source |
|---|---|---|
| 23 | 1 | PS(1) |
| 24 | 2 | 40%N(1), PS(1) |
| 25 | 1 | 40%N(1) |
| 26 | 0 | |
| 27 | 3 | CBS(1), CW(1), $CW_{50}$(1) |
| 28 | 2 | $CS_{50}$(2) |
| 29 | 1 | 10%(1) |
| 30 | 3 | CBS(2), $CW_{100}$(1) |
| 31 | 5 | PW(1), PS(1), $CW_{50}$(2), $CW_{100}$(1) |
| 32 | 2 | NWD(2) |
| 33 | 2 | PW(2) |
| 34 | 2 | CBS(2) |
| 35 | 3 | CW(3) |
| 36 | 1 | NWD(1) |
| *Sarcinia lutea* | 8 | 40%(1), PW(1), $CW_{50}$(1), $CW_{100}$(5) |
| *Alteromonas rubra* | | Csh(2) |
| *A. luteo-violaceus* | | Csh(1), P40(1), C40(2) |
| Cytophaga | | C40(2), Cg(3) |

[1]Key to Source Codes

Water samples:

- PW = Pier 3 water
- CW = Control site water
- $CW_{50}$ = Control site 50 meters offshore
- $CW_{100}$ = Control site 100 meters offshore

Sediment samples:

- PS = Pier 3 sediment
- CS = Control site sediment

Sediment Samples - continued

$CS_{50}$ = Control site sediment 50 M offshore

$CS_{100}$ = Control site sediment 100 M offshore

Piling samples:

40%N = 40% naphthalene fender piling-intertidal

1↑ = 40% naphthalene fender piling-1 M above water

1↓ = 40% naphthalene fender piling-1 M below water

SCR = 40% naphthalene fender biotic layer

40% = 40% naphthalene piling in place 1 year - intertidal

10% = 10% naphthalene piling in place 1 year - intertidal

NWD = 30% naphthalene piling in place 1 year - intertidal

OWD = Pier 2 sample - intertidal

Test surface samples:

Pg = Pier 3 glass

Psh = Pier 3 shell

P40 = Pier 3 40% naphthalene wood

Cg = Control site glass

Csh = Control site shell

C40 = Control site 40% naphthalene wood

Table 2. Differential characters of the diagnostic key employed for rapid identification of bacteria isolated from samples collected at Roosevelt Roads, P.R.

| |
|---|
| Spreading colonies |
| Gram Stain |
| Fermentative (F) - |
| Oxidative (O) |
| Casein hydrolysis |
| Gelatin hydrolysis |
| $H_2S$ production |
| Methyl-red test |
| $NO_3 \rightarrow NO_2$ |
| Tween 40 hydrolysis |
| Tween 60 hydrolysis |
| Tween 80 hydrolysis |
| Xanthine degradation |
| Growth at: |
| NaCl (7.5% $^{w}/_{v}$) |
| pH 8 |
| Utilization of: |
| Cellobiose |
| Fructose |
| Inulin |
| Proline |
| Sodium acetate |

Table 3. Seasonal occurrence and distribution of major bacterial phena comprising microbial communities in water, sediment, and pilings at Roosevelt Roads, P.R.

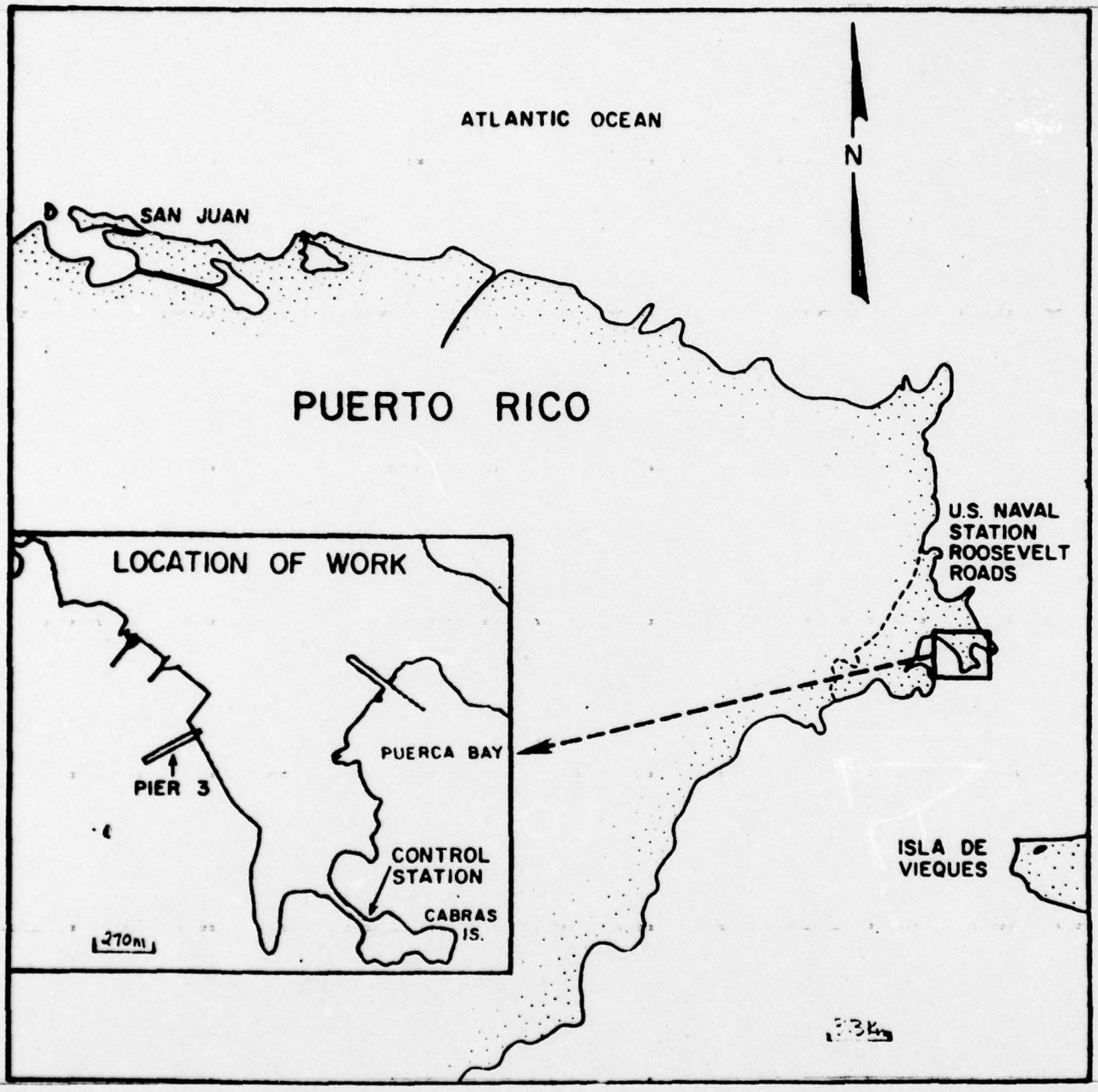
| October 1977[a] Phenon | Presumptive identification | Isolates at Pier 3 | Isolates at Control site | Total # of isolates |
|---|---|---|---|---|
| 13 | *Bacillus megaterium* | 75 | 10 | 85 |
| 17 | Unidentified sheathed bacterium | 45 | 4 | 49 |
| 22 | *Hyphomicrobium vulgare* | 22 | 0 | 22 |
| 2 | Gram negative rods | 20 | 0 | 20 |
| 4 | *Vibrio* sp. | 11 | 0 | 11 |
| June 1978 | | | | |
| 13 | *Bacillus megaterium* | 0 | 0 | 0 |
| 17 | Unidentified sheathed bacterium | 49 | 0 | 40 |
| 22 | *Hyphomicrobium vulgare* | 27 | 8 | 35 |
| 2 | Gram negative rods | 14 | 16 | 30 |
| 4 | *Vibrio* sp. | 39 | 9 | 48 |

[a] (Salinity = 35‰ , Temp 29°C).

Table 4. Characteristics of new phena isolated at Roosevelt Roads during June 1978 sampling trip

| Phenon | |
|---|---|
| 32 | Gram-negative, oxidative, motile, catalase and oxidase positive rods, reducing $NO_3$ to $NO_2$, degrading xanthine but not casein, gelatin, and Tween 40, 60 and 80, $H_2S$, was not produced. Cellobiose, fructose, inulin, proline and sodium acetate were not utilized. |
| 33 | Gram-negative, oxidative, motile, catalase and oxidase positive rods, which degrade casein, gelatin and Tween 80, and utilize inulin and proline. |
| 34 | Gram-negative, oxidative, catalase and oxidase positive rods which degrade Tween 40, 60 and 80, and xanthine. |
| 35 | Gram-negative, fermentative, oxidase positive, but catalase negative rods, which degrade casein, gelatin, Tween 40, 60 and 80, and xanthine, and reduce nitrate to nitrite. |
| 36 | Gram-negative, catalase-positive, oxidative rods, which degrade Tween 40, 60 and 80, and utilize sodium acetate. |

Fig. 1 Location of sampling stations at Roosevelt Roads, Puerto Rico.


ATLANTIC OCEAN
N
SAN JUAN
PUERTO RICO
U.S. NAVAL STATION ROOSEVELT ROADS
LOCATION OF WORK
PUERCA BAY
PIER 3
CONTROL STATION
CABRAS IS.
270m
ISLA DE VIEQUES
33Km